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Effects of ascorbic acid and ferrous sulfate on trace element extractability by dialyzation of weaning foods

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Abstract

Response surface methodology was used to study the effects of pea and soybean, and the effects of fortifying weaning foods with ascorbic acid and ferrous sulfate, on the dialyzabilities of copper, zinc and iron. Of the three elements, only zinc was insensitive to all four factors. The marked influence of pH on dialyzed iron is related to the ability of certain ingredients to alkalinize (soybean) or acidify (ascorbic acid) food. Under the conditions tested, using ready-to-eat foods, the negative effect of soybean on trace element dialyzability was significant only in the case of iron. Although ascorbic acid had a positive effect on iron dialyzability, it also had a strong negative effect on that of copper. Fortification using ferrous sulfate elicited a similar positive effect on dialyzabilities of both copper and iron.

Keywords: Infant foods; Trace elements; Dialyzability; Ascorbic acid; Ferrous sulphate

1. Introduction

Pediatric guidelines recommend that beikosts should gradually replace exclusive milk feeding from the fifth month of life onwards (Kersting, Kaiser, & Schoch, 1995). At this age, most infants begin to eat supplementary semi-solid foods, and weaning foods or beikosts therefore play a major role in their nutrition. The term "beikost" has been defined as "any additional food used in infant nutrition other than human milk and formulas" (Fomon, 1974).

Several factors have been reported to inhibit trace element absorption. Phytic acid, for example, can reduce the bioavailability of certain trace elements such as zinc (−62%), iron (−48%) or copper (−31%) (Lopez, Coudray, Levrat-Verny, Feillet-Coudray, Demigné, & Rémésy, 2000); tannins have been found to depress nonheme iron absorption (Cook, Reddy, & Hurrell, 1995); studies addressing the influence of fibre on trace element absorption have yielded conflicting results, but there is evidence to support in vitro inhibition (Carnovale & Lintas, 1995). Although certain dietary factors appear to affect trace element (e.g. iron) absorption when examined individually, their influence largely disappears in multiple regression analysis (Reddy, Hurrell, & Cook, 2000); these factors should therefore be studied in ready-to-eat foods rather than in single ingredients in isolation.

Although the in vivo system is considerably more complex, in vitro studies of mineral binding may serve to identify the mechanisms involved. Prior to undertaking in vivo bioavailability studies in children, therefore, it would appear wise to perform in vitro studies using a dialyzability or Caco-2 cell uptake model; this ensures a swifter and less costly approach, given that human studies require plant foods to be labeled intrinsically with either stable isotopes or radioisotopes. In vitro testing of this sort may afford a useful preliminary screening tool for predicting the bioavailability of trace elements.

Response surface methodology (RSM) is a very useful statistical tool, comprising a set of techniques used in the empirical study of relationships between one or more responses and a group of input variables, in order to locate the region of lowest response values, where the

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lowest is considered to be the best (Myers & Montgomery, 1995).

The aim of this study was to determine the best formulation of different ingredients with a view to increasing dialyzable Cu, Zn and Fe in weaning foods.

2. Material and methods

2.1. Infant food samples

Beikost samples (250 g bottles) were manufactured by Hero España S.A., using different formulations of several ingredients in accordance with the experimental design described below. The manufacturing process has been previously described (Ros, Abellán, Rincón, & Periago, 1994).

2.2. Experimental design and statistical analysis

The following constant ingredients were formulated: meat (lamb), carrot, potato, onion, sunflower oil and water. In addition, two key ingredients, pea and soybean, were selected for reasons detailed in earlier papers (Abellán, Rincón, Ros, & López, 1994; Rincón, Ros, Periago, & Martínez, 1996). Both ascorbic acid and ferrous sulfate were included in the product formula, because apart from phytic acid, these are the major factors for predicting non-heme iron absorption (Reddy et al., 2000). The food formulation process can therefore be considered as a system including four *input* factors (ingredients included in the experimental design, as shown in Table 1) and twelve *output* factors (responses). The general design was a second-order full factorial design, including three levels for each factor, following a previously described model (Box & Behnken, 1960). Full design is shown in Table 2.

Each response-variable was assumed to be influenced by four independent variables or factors (pea, soybean, ascorbic acid and ferrous sulfate), Φ_i (i = from 1 to 4), so that $\zeta_m = f(\Phi_1, \Phi_2, \Phi_3, \Phi_4)$, where ζ is each response (m = from 1 to 12), Φ_1 is the percentage of pea formulated, Φ_2 is the percentage of soybean formulated, Φ_3 is the percentage of ascorbic acid formulated, and Φ_4 is the percentage of ferrous sulfate formulated.

Table 2
Experimental design in both standard (run row) and randomized (trial row) orders

Run	Trial	Factors			
		X_1	X_2	X_3	X_4
1	25	−1	−1	0	0
2	23	−1	0	−1	0
3	11	−1	0	0	−1
4	24	−1	0	0	1
5	3	−1	0	1	0
6	1	−1	1	0	0
7	9	0	−1	−1	0
8	6	0	−1	0	−1
9	13	0	−1	0	1
10	18	0	−1	1	0
11	10	0	0	−1	−1
12	12	0	0	−1	1
13	16	0	0	0	0
14	2	0	0	1	−1
15	7	0	0	1	1
16	15	0	1	−1	0
17	8	0	1	0	−1
18	21	0	1	0	1
19	4	0	1	1	0
20	19	1	−1	0	0
21	22	1	0	−1	0
22	14	1	0	0	−1
23	17	1	0	0	1
24	5	1	0	1	0
25	20	1	1	0	0

Factors X_1, X_2, X_3 and X_4 represent percentages of pea, soybean, ascorbic acid and ferrous sulphate, respectively, according to the experimental conditions shown in Table 1.

The basic analysis for a response surface experiment consists of fitting a quadratic model of the form

$$\xi = b_0 + \sum_{i=1}^{p} b_i X_i + \sum_{i=1}^{p} b_{ii} X_i^2 + \sum_{i=1}^{p-1} \sum_{j=i+1}^{p} b_{ij} X_i X_j + \varepsilon, \quad (1)$$

where ζ is each response, X_i are factors or key ingredients considered for each beikost type, as coded independent variables, since it is advisable to transform natural variables into coded variables, which are usually defined as dimensionless with mean zero and the same spread or standard deviation (Myers & Montgomery, 1995); X_iX_j are the two factor interactions, b_0 is the intercept, b_i, b_jb_{ij} are linear, quadratic and cross product regression terms, respectively and ε the model error. In order to describe each response-factor relationship by a

Table 1
Levels of key ingredients as % considered in the experimental design

Factors	Symbol			Levels		
	Unit	Coded	Uncoded	−1	0	+1
Pea	%	X_1	Φ_2	3.5	5.0	6.50
Soybean	%	X_2	Φ_2	2.0	3.0	4.00
Ascorbic acid	%	X_3	Φ_2	0.02	0.04	0.06
Ferrous sulfate	%	X_4	Φ_2	0.005	0.010	0.015

polynomial equation with squared terms, trials at three levels of variable formulation were required; the levels considered are shown in Table 1.

The twelve responses measured were: phytic acid, tannin, insoluble, soluble and total dietary fibre, Cu, Zn, Fe, dialyzed Cu, Zn and Fe, and pH. The Design-Expert software package (Stat-Ease, Inc., Minneapolis) was used to generate designs, fit the response surface model to the experimental data and draw response surface figures. Differences were considered significant at $p \leqslant 0.05$ level.

2.3. Methods

Phytic acid was extracted by following the method of Plaami and Kumpulainen (1991); phytic phosphorus was then determined by the standard method 970.39 (AOAC, 1990). Total dietary fibre (TDF), soluble dietary fibre (SDF) and insoluble dietary fibre (IDF) were determined using the method described by Prosky, Aspm, Schweizer, Devries, and Furda (1988). A specific method covering a wide range of ingredients of vegetable origin has been described for determination of tannins in infant foods (Martinez, Rincón, & Ibáñez, 2000).

The Cu, Fe and Zn content of weaning food were analyzed after destruction of organic matter by the dry method. Samples (50 g) were first dried overnight in a forced-draft oven at 90 °C. Ashing was carried out in a Nabertherm furnace oven, model L3/P (Lilienthal, Bremen, Germany), applying the following mineralization stages to prevent mineral loss by volatilization: 90–200 °C (ramp time 1 h, hold time 2 h); 200–450 °C (ramp time 2.5 h, hold time 16 h) and 450–100 °C (ramp time 2 h). To check that there was no loss of mineral elements, recovery studies were done in incinerated samples at different temperatures/times; the best recovery percentages were obtained at 450 °C. The ash was dissolved with 2 ml HNO_3 and the solution obtained was evaporated on a thermostatic plate without reaching the boiling point of the acid mixture, to avoid sample loss through splashing, and placed again in the furnace oven (450 °C) for 1 h. White ash was subsequently collected with 5 ml of HNO_3, making up a final volume of 50 ml with distilled deionised water. Analytical determinations were performed with a Perkin–Elmer model 2380 atomic absorption spectrophotometer equipped with a Perkin–Elmer AS-50 autosampler. A 10 cm one-slot burner head with standard air–acetylene flame and single element hollow cathode lamps was employed for elemental analyses. Sodium and potassium determinations were carried out by flame atomic emission spectrophotometry. The nitric acid solution (Suprapur® quality) and the mineral standard solution (Tritisol®) used were obtained from Merck (Darmstadt, Germany).

Dialyzable Cu, Zn and Fe were determined using the technique described by Miller, Schricker, Rasmussen, and Van Campen (1981) and later modified by Vaquero, Van Dokkum, Bos, Wolters, Schaafsma, and Luten (1992).

3. Results and discussion

3.1. General

Table 3 shows the results obtained for each trial, and Table 4 the significant effects ($p \leqslant 0.05$) of each ingredient (as coded independent variable X_1 to X_4) on each response (Y_1 to Y_{12}). All main effects (originating from each single factor separately) were linear effects (Table 3). In addition, some secondary effects (from the interaction of two factors) on Y_9 (dialyzed Zn) were obtained: $X_1 \times X_4$ (−3.16), $X_1 \times X_2$ (−3.02) and $X_1 \times X_3$ (−2.70).

3.2. Phytic acid

Both soybean and pea showed significant positive effects on phytic acid content, that of soybean doubling that of pea (Table 4), despite accounting for a smaller proportion of the total formulation (Table 1). This may explain why increasing the proportion of pea from 3.50% to 6.5% (Table 1) did not prompt any significant decline in the dialyzable amount of any trace element, whilst the presence of soybean did affect dialyzable iron ($p \leqslant 0.05$, Table 4), though not copper ($p \leqslant 0.65$). Other authors also report that phytic acid does not inhibit absorption either of copper (Lönnerdal, 2002) or of zinc ($p \leqslant 0.26$) although, in the latter case, the inhibitory effect depends on the phytate/zinc ratio (Morris & Ellis, 1980). These results support the view that the inhibitory effect of phytate is stronger for iron than for zinc (Lönnerdal, 2002), possibly because, in addition to the negative effect of phytic acid, soybean (*Glycine max*) contains a protein-related moiety in the 7S protein (conglydinin) that depresses Fe absorption (Lynch, Dassenko, Cook, Juillerat, & Hurrell, 1994).

Two hypotheses may be adduced to account for these results: first, pea phytic acid is chemically different from soybean phytic acid, and thus differs in terms of its affinity for divalent cations and thence it antinutritive effect; second, phytic acid may not have the same affinity for Fe^{3+}, Fe^{2+}, Zn^{2+} and Cu^{2+}. With regard to the first hypothesis, it must be borne in mind that hexa-myoinositol and penta-myoinositol make up over 90% of the total phytate in raw grains (Agte, Tarwadi, & Chiplonkar, 1999) but are degraded during processing and cooking; when phytic acid (hexa-myoinositol, IP6) is dephosphorylated, other isomers of lower inositol phosphates such as myoinositol penta- (IP5), tetra- (IP4), tris- (IP3) or bi- (IP2) are obtained (Torre,

Table 3
Results obtained for the different responses in weaning food[a]

Run	Y_1	Y_2	Y_3	Y_4	Y_5	Y_6	Y_7	Y_8	Y_9	Y_{10}	Y_{11}	Y_{12}
1	1.47	0.27	7.67	1.81	9.48	0.070	2.03	0.758	12.1	1.84	19.4	5.89
2	1.58	0.58	7.94	1.77	9.71	0.097	2.81	0.620	12.4	1.65	15.2	5.97
3	1.60	0.11	6.99	0.33	7.32	0.097	2.02	0.688	14.4	1.27	12.0	5.92
4	1.68	0.45	8.67	0.79	9.46	0.093	2.75	0.734	19.6	2.25	19.3	5.92
5	1.51	1.13	8.04	1.13	9.17	0.118	2.10	0.854	19.7	2.08	21.3	5.91
6	1.79	0.39	8.36	0.84	9.20	0.125	2.11	0.583	19.3	1.53	17.3	5.96
7	1.27	0.32	8.16	1.13	9.29	0.084	3.51	0.599	17.0	1.75	15.5	5.96
8	1.71	0.73	6.57	1.15	7.72	0.097	2.15	0.667	17.5	1.59	17.9	5.89
9	1.66	0.77	7.59	1.39	8.98	0.091	3.07	0.700	16.9	2.19	22.0	5.88
10	1.36	0.71	8.89	1.98	10.9	0.096	2.15	0.607	16.8	1.85	18.5	5.92
11	1.81	0.89	9.04	1.52	10.6	0.086	3.20	0.659	17.2	1.84	13.2	6.00
12	1.82	0.19	6.13	1.56	7.69	0.110	3.89	0.722	17.2	2.75	16.4	6.05
13	1.72	0.61	8.71	1.58	10.3	0.112	2.60	0.728	16.6	2.32	16.5	5.93
14	1.83	1.63	7.56	1.45	9.01	0.105	2.25	0.773	16.1	1.67	15.8	5.91
15	1.74	1.63	6.57	1.69	8.26	0.101	2.12	0.722	14.6	2.54	18.1	5.89
16	1.88	1.12	7.30	1.78	9.08	0.111	2.86	0.707	17.6	2.42	15.5	6.00
17	2.07	0.82	10.67	2.26	12.9	0.113	2.00	0.807	17.1	2.01	15.4	5.94
18	2.22	0.74	8.60	2.85	11.5	0.125	3.86	0.778	18.4	2.72	19.6	5.95
19	2.07	0.60	8.13	1.35	9.48	0.064	2.08	0.690	17.5	1.86	19.2	5.89
20	1.68	0.79	9.40	2.51	11.9	0.075	2.83	0.835	17.5	2.15	21.1	5.89
21	1.83	0.86	7.58	1.41	8.99	0.138	3.68	0.632	19.7	2.33	18.9	5.94
22	1.90	1.39	9.15	1.90	11.05	0.095	2.59	0.610	19.1	1.72	15.3	5.90
23	2.05	0.69	5.48	1.66	7.14	0.117	2.55	0.740	13.8	2.59	18.1	5.89
24	1.73	0.55	7.37	1.66	9.03	0.098	2.44	0.619	18.0	2.27	18.9	5.84
25	2.03	0.84	8.27	0.94	9.21	0.124	2.23	0.662	14.7	2.43	15.2	5.91

[a] Y_1 = phytic acid (mg/g), Y_2 = tannin (catechin U/g), Y_3 = insoluble dietary fibre IDF (%), Y_4 = soluble dietary fibre SDF (%), Y_5 = total dietary fibre TDF (%), Y_6, Y_8 and Y_{10} = copper, zinc and iron content (mg/100 g), Y_7, Y_9 and Y_{11} = dialyzed copper, zinc and iron (%), respectively, and Y_{12} = pH. All results are expressed on a wet weight basis.

Table 4
Significant ($p \leqslant 0.05$) standardized main effects for each ingredient considered as factors on the responses evaluated in the final ready-to-eat product

Response[a]	X_1c		X_2		X_3		X_4	
	Linear	Quadratic	Linear	Quadratic	Linear	Quadratic	Linear	Quadratic
Y_1	4.22		7.70					
Y_2								
Y_3			2.97					
Y_4								
Y_5			2.25					
Y_6			2.59					
Y_7					−5.23		3.09	
Y_8								
Y_9								
Y_{10}	4.21		2.36				7.20	
Y_{11}			−2.03		2.79		3.91	
Y_{12}	−2.40		2.64		−6.71			

[a] Y_1 = phytic acid (mg/g), Y_2 = tannin (catechin U/g), Y_3 = insoluble dietary fibre IDF (%), Y_4 = soluble dietary fibre SDF (%), Y_5 = total dietary fibre TDF (%), Y_6, Y_8 and Y_{10} = copper, zinc and iron content (mg/100 g), Y_7, Y_9 and Y_{11} = dialyzed copper, zinc and iron (%), respectively, and Y_{12} = pH. All results are expressed on a wet weight basis.

Rodriguez, & Saura-Calixto, 1991); studies have shown that the maximum amount of metal ion bound by the various inositol phosphates is approximately the same for IP6 and IP5 but decreases upon further dephosphorylation (Persson, Türk, & Nyman, 1998) and, for example, inositol phosphates lower that IP5 do not inhibit non-heme iron absorption (Allen & Ahluwalia, 1997). With regard to the second hypothesis, in the pH range 3.5–7 (in the present study pH ranged from 5.84 to 6.05, Table 3), binding strength is the same for all inositol phosphates, i.e. $Cu^{2+} > Zn^{2+}$ (Persson et al., 1998), although in myoinositol triphosphates complex stability has been shown to follow the order $Cu^{2+} > Zn^{2+} > Fe^{2+}$ (Mernissi-Arifi, Wehrer, Schlewer, & Spiess, 1994). However, rather than food, both of these studies used solutions of sodium phytate hydrolyzed with HCl and various myoinositol reagents, respectively. By contrast, the results obtained here with ready-to-eat food indicate

that phytic acid binds better to iron than to zinc or copper, suggesting that under the pH conditions used here, most iron is present in ferrous form; hence, phytic acid shows greater affinity (Mernissi-Arifi et al., 1994). Since phytic acid binds metallic cations via ionic associations with negatively charged phosphates (Lott, Greenwood, & Batten, 1995), the fall in pH prompted by ascorbic acid would account for the positive effect observed on dialyzable iron, the circumstances favouring conversion of Fe^{3+} to Fe^{2+}.

Of all food composition factors, most of the total variance in non heme iron absorption is explained by phytic acid content (Reddy et al., 2000); phytin-containing particles, called globoids, found in the scutellum and aleurone layer, are determined genetically in all rice varieties by the cDNA clone pRINO1 (Yoshida, Wada, Koyama, Mizobuchi, & Naito, 1999). Genetic engineering techniques have therefore been proposed as a way of reducing the concentration of substances which inhibit trace element absorption (Frossard, Bucher, Mächler, Mozafar, & Hurrell, 2000); such techniques have been successfully used to reduce phytic acid in cereal (Adams et al., 2002), and these cultivars should be used as ingredients of infant foods.

3.3. Tannins

With the pea and soybean proportions used here (3.5–6.5% and 2–4%, respectively, Table 1), no significant effect was observed on tannin content (Table 4), suggesting that neither ingredient would effectively control tannin content in the infant foods studied. Although many authors report a negative effect of various polyphenols on trace element absorption (Frossard et al., 2000), Reddy et al. (2000) concluded that certain dietary factors were correlated with trace element absorption when examined individually, but that their influence largely disappeared in multiple regression analysis; polyphenols, for example, were not significant in predicting iron absorption from meals containing several food items.

3.4. Fibre

Both IDF and TDF were affected by soybean (Table 4); correlation between the two was significant ($r = 0.99$, $p \leqslant 0.05$), so both observed effects were redundant. Pea was present in a higher proportion than soybean (Table 1), but exerted no significant effect (Table 4).

3.5. Cu and dialyzed Cu

Soybean recorded a significant effect on copper levels in food (Table 4).

Contrary to expectations, neither pea nor soybean showed significant effects on dialyzable copper, even though both increased the amount of phytic acid in the food (Table 4), suggesting that in the presence of other cations, phytic acid shows low affinity for Cu^{2+}; this once again highlights the considerable disparity between the results obtained using reagent solutions (Mernissi-Arifi et al., 1994; Persson et al., 1998) and those obtained using food, a disparity recently signaled in a paper studying the inhibitory effect of polyphenols on iron absorption (Reddy et al., 2000). Although the results obtained in the present study agree with those recently reported by López et al. (2000) and Lönnerdal (2002), who conclude that phytic acid does not inhibit copper absorption, they are nonetheless difficult to account for in view of the considerable affinity of inositol forms (IP6 to IP3) for Cu^{2+} (Persson et al., 1998), unless the results obtained by these authors in aqueous solutions cannot be extrapolated to complete foods, a finding reported for in vivo experiments (Reddy et al., 2000). Recent studies have shown that phytic acid does not have a significant depressive effect on copper status in rats (López et al., 2000).

With regard to the negative effect of ascorbic acid (−5.23, Table 4), similar findings are reported by other authors (Fairweather-Tait, 1992). Ascorbic acid displayed a highly negative effect (−6.71) on pH (Table 4), modifying this response (Y_{12}) in the range 6.05–5.84 (Table 3); in this range there is greater binding of metal by phytic acid, and especially IP3 and IP4 (Persson et al., 1998), so the effect of ascorbic acid on the percentage of dialyzable copper may be due to its effect on pH (Table 4). The positive effect of ferrous sulfate on dialyzable copper (Table 4) suggests a stronger affinity of phytic acid for Fe, which only occurs if iron is present in the form of Fe^{3+} (Mernissi-Arifi et al., 1994). Since the presence of both ferrous (Fe^{2+}) and ferric (Fe^{3+}) is dependent on pH, the effect of ascorbic acid on pH (Table 4) may account for the positive effect of $FeSO_3$ on copper dialyzability if a higher ferric form is produced. Given the negative effect of ascorbic acid on dialyzable copper, its positive effect on dialyzable iron and the fact that dialyzable zinc is not sensitive to ascorbic acid, a certain percentage of ascorbic acid may be considered useful in order to obtain an acceptable percentage of dialyzable copper and iron (Fig. 1).

3.6. Zn and dialyzed Zn

None of the factors displayed any effect on either zinc content or percentage of dialyzable zinc (Table 4). These results appear unusual, since many authors report a negative correlation between phytate content and zinc absorption; both ingredients showing a significant effect on phytic acid content (pea and soybean, Table 4) would be expected to have a negative effect on Zn dialyzability, yet neither the increase in pea proportions ($p \leqslant 0.37$) from 3.5% to 6.5% (Table 1) nor that of soybean

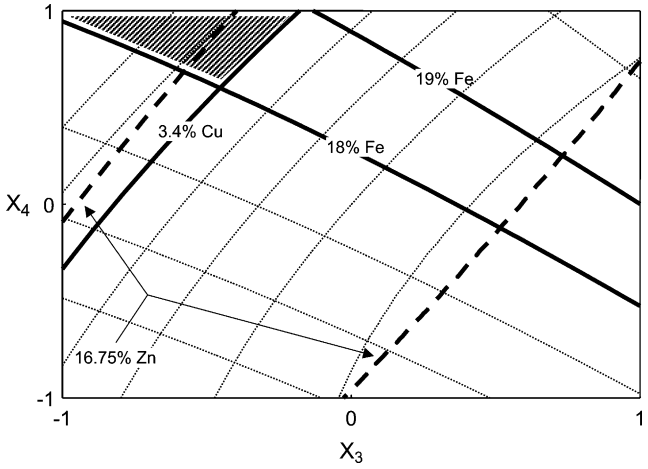

Fig. 1. Highest simultaneous dialyzable iron, copper and zinc percentages (shaded area) as a function of ascorbic acid (X_3) and ferrous sulfate (X_4). Dialyzable zinc percentage was not sensitive to both factors.

proportions ($p \leqslant 0.26$) from 2% to 4% (Table 1) exerted statistically significant effects (Table 4). To account for these apparently unusual results, it must be borne in mind that a higher affinity of phytic acid species has been reported for Cu^{2+} and that there is saturation of available binding sites on inositol phosphates at pH 5–6 (Persson et al., 1998); this, together with the stronger affinity of phytic acid for Fe^{3+} than Fe^{2+} (Reddy et al., 2000) may account for these results (Table 4). Since a higher phytic acid content is also correlated with higher copper content in weaning food, in the study conditions applied here dialyzable Zn would remain insensitive to the effects of phytic acid. This hypothesis, and the fact that in the pH range from 5.5 to 7.0, an increase of one pH unit causes a 30–45-fold decrease in aqueous Zn^{2+} ion concentrations and 100- and 1000-fold decrease in aqueous Fe^{2+} and Fe^{3+} ion concentrations (Frossard et al., 2000), might account for similar findings obtained by other authors in soy-based infant formulas (Jovani, Alegría, Barberá, Farre, Lagarda, & Clement, 2000).

3.7. Fe and dialyzed Fe

Inclusion of an iron supplement (ferrous sulfate from 0.005% to 0.015%) yielded an effect (7.20) similar to that obtained by joint inclusion of pea (4.21) and soybean (2.36) (Table 4) simultaneously (6.57). Thus, any fortification strategy in food of this type must include a ferrous sulfate percentage higher than those assayed here in order to ensure an effect significantly greater than those obtained by the two vegetable ingredients.

A significantly higher mean percentage (17.4%) was obtained for dialyzable iron than in other weaning foods based on meat, such as chicken (10.3%) or veal (9.40%) (data not shown). These differences are clearly due to ferrous sulfate fortification since, while other meat-based infant foods contain 20% meat (ESPGAN, 1981), lamb-based foods contain only 15% meat, because a very intense lamb flavour is not acceptable in the Spanish market (Abellán et al., 1994); moreover, the iron content of lamb is lower than that of veal or chicken (Souci, Fachmann, & Kraut, 1994).

Both ascorbic acid and ferrous sulfate showed significant positive effects on dialyzable iron (2.79 and 3.91, respectively), but soybean displayed a negative effect (−2.03, Table 4). Since polyphenols are known to be of little or no significance in predicting iron absorption (Reddy et al., 2000), and since soybean here displayed no significant effect on tannin content (Table 4) in the experimental range under study (Table 1), the negative effect of soybean on dialyzable iron can be entirely attributed to phytic acid, although other authors report an inhibitory effect of soy protein on iron absorption in infant formulas (Jovani et al., 2000). However, both soybean and ascorbic acid showed inverse significant effects (Table 3) on both pH (Y_{12}) and dialyzed iron (Y_{11}). As Fig. 2 shows, there was a considerable overlap of % dialyzed iron and pH affected by soybean and ascorbic acid; the negative effect of soybean on iron dialyzability, and thus its antinutritive effect, must therefore be supported by both the increased phytic acid content and the significant alkalization of the medium (Table 4). This may account for the conflicting results published by other authors: while some conclude that most tannin resides in the seed coat and acts as a potent inhibitor of Fe (Gillooly et al., 1984), others report that the soybean seed coat contains relatively high levels of bioavailable iron (Laszlo, 1991), and that no correlation exists between levels on increased phytase treatment and iron dialyzability (Lombardi-Boccia, Martínez, Aguzzi, & Rincón, 2002). These substantial differences are in all likelihood due to the different pH levels at which the

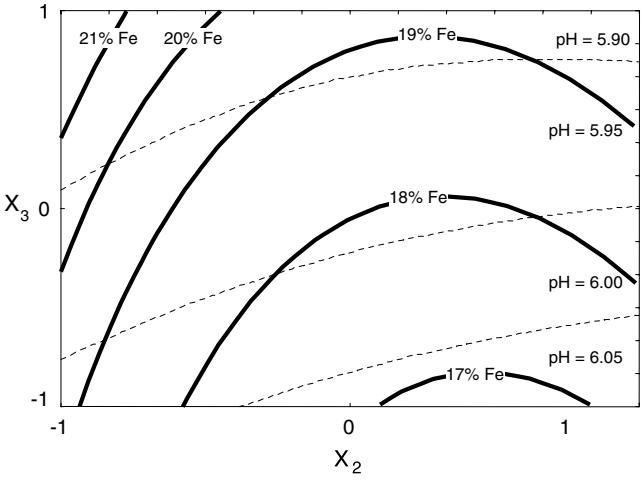

Fig. 2. Simultaneous effects of X_2 (soya) and X_3 (ascorbic acid) on Y_{11} and Y_{12} (dialyzed iron and pH, respectively) at $X_1 = 0$ and $X_4 = -1$.

various studies were performed, since variation of pH, even within a narrow range (5.90–6.05, Fig. 2), can have a significant effect on iron dialyzability, and presumably on iron absorption, given that the pH of the small and large intestine is around 6 (Persson, Nyman, Liljeberg, & Frølich, 1991).

Soybean is also reported to contain other antinutritive factors, in addition to phytic acid, that affect iron, but not zinc or copper, dialyzability; there is, for example, a protein-related moiety in the 7S protein (conglydinin) that depresses Fe absorption (Lynch et al., 1994) and, in soybean-meal extracts, more of the Fe appears to be bound directly to protein rather than through phytic acid (Honing & Wolf, 1991). Further research is therefore required to accurately apportion the relative contributions of phytic acid and soybean protein to inhibition of iron absorption.

The enhancing effect of ascorbic acid, by maintaining the solubility of nonheme iron when the food enters the alkaline environment of the small intestine, has long been recognized as reducing the influence of the inhibitory ligands that bind iron in the more alkaline pH of the duodenum (Hurrell, 1984). Fig. 2 also shows that the beneficial effect of ascorbic acid on iron dialyzability is in great measure due to acidification of the medium (Table 4); it might thus be hypothesized that other acidifying agents, acting at local level in the duodenum, exert the same beneficial effect on iron absorption as ascorbic acid. The positive effect of ascorbic acid on iron dialyzability (Table 4) may thus be attributed to acidification of the medium (Fig. 2), and to the fact that, at low pH, the chemical oxidation of ferrous iron (Fe^{3+}) to ferric iron (Fe^{2+}) is very low. Under these conditions, phytic acid chelates primarily to Cu^{2+} (Persson et al., 1998), which would account for the negative effect of ascorbic acid on copper dialyzability (Table 4). In addition, the principal transporter mediating non-heme iron uptake by the enterocyte is thought to be the divalent metal transporter (DMT1), which requires acidic conditions in the proximal duodenum to bind Fe^{2+} (Minihane & Rimbach, 2002).

To summarize, use of response surface methodology has shown that soybean has a stronger effect than pea on iron dialyzability, with no significant effect on zinc or copper under the study conditions applied here. The results suggest that these effects are closely linked to the pH of the food, which is thus a major parameter for determining trace element bioavailability; the influence of subsequent variations in pH during digestion is less marked.

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References

Abellán, P., Rincón, F., Ros, G., & López, G. (1994). Mineral composition of meat-based infant beikosts. A preliminary study. *International Journal of Food Science & Nutrition, 45*, 209–215.

Adams, C. L., Hambidge, M., Raboy, V., Dorsch, J. A., Sian, L., Westcott, J. L., & Krebs, N. F. (2002). Zinc absorption from a low-phytic acid maize. *American Journal of Clinical Nutrition, 76*, 556–559.

Agte, V. V., Tarwadi, K. V., & Chiplonkar, S. A. (1999). Phytate degradation during traditional cooking: significance of the phytic acid profile in cereal-based vegetarian meals. *Journal of Food Composition & Analysis, 12*, 161–167.

Allen, L.H., Ahluwalia, N. (1997). Bioavailability of nonheme iron. In: L. H. Allen, N. Ahluwalia (Ed.), Improving iron status through diet. The application of knowledge concerning dietary iron bioavailability in human populations. US Agency for International Development, Arlington, VA. Final Report of the Contract no. HRN-5122-C-00-3025-00, John Snow, Inc./OMNI Project, pp. 13–34.

AOAC. 1990. *Official methods of analysis.* 15th ed. Washington, DC: Association of Official Analytical Chemists, The Association.

Box, G. E. P., & Behnken, D. W. (1960). Some new three level designs for the study of quantitative factors. *Technometrics, 2*, 455–475.

Carnovale, E., & Lintas, C. (1995). Dietary fibre: effect of processing and nutrient interactions. *European Journal of Clinical Nutrition, 49*(Suppl. 3), S307–S311.

Cook, J. D., Reddy, M. B., & Hurrell, R. F. (1995). The effect of red and white wines on nonheme iron absorption in humans. *American Journal of Clinical Nutrition, 61*, 800–804.

ESPGAN (1981). European Society of Pediatrics, Gastroenterology and Nutrition – Committee on Nutrition. Guidelines on infant nutrition. II. Recommendations for the composition of follow-up formula and beikost. *Acta Paediatrica Scandinavica,* (Suppl. 287), 1–25.

Fairweather-Tait, S. (1992). Bioavailability of trace elements. *Food Chemistry, 43*, 213–217.

Fomon, S. J. (1974). *Infant nutrition* (2nd ed.). Philadelphia, Pennsylvania, USA: W.B. Saunders.

Frossard, E., Bucher, M., Mächler, F., Mozafar, A., & Hurrell, R. (2000). Potential for increasing the content and bioavailability of Fe, Zn, and Ca in plants for human nutrition. *Journal of the Science of Food & Agriculture, 80*, 861–879.

Gillooly, M., Bothwell, T. H., Charlton, R. W., Torrance, J. D., Bezwoda, W. R., McPhail, E., & Derman, D. P. (1984). Factors affecting the absorption of iron from cereals. *British Journal of Nutrition, 51*, 37–46.

Honing, D. H., & Wolf, W. J. (1991). Phytate–mineral–protein composition of soybeans: Gel filtration studies of soybean meal extracts. *Journal of Agriculture & Food Chemistry, 39*, 1037–1042.

Hurrell, R. (1984). Bioavailability of different iron compounds used to fortify formulas and cereal: technological problems. In A. Stekel (Ed.), *Iron nutrition in infancy and childhood* (pp. 147–178). New York: Raven Press.

Jovani, M., Alegría, A., Barberá, R., Farre, R., Lagarda, M. J., & Clement, G. (2000). Effect of proteins, phytates, ascorbic acid and citric acid on dialysabitity of calcium, iron, zinc and copper in soy-based infant formulas. *Nahrung, 44*(2S), 114–117.

Kersting, M., Kaiser, B., & Schoch, G. (1995). Nutrition of infants between 5th and 12th month of life. *Ernahrungs-Umschau, 42*(1), 18–21.

Laszlo, J. A. (1991). Changes in endogenous and exogenous iron-reducing capability of soybean hull during development. *Cereal Chemistry, 68*, 21–24.

Lombardi-Boccia, G., Martínez, B., Aguzzi, A., & Rincón, F. (2002). Optimization of heme iron analysis in raw and cooked red meat. *Food Chemistry, 78*, 505–510.

Lönnerdal, B. (2002). Phytic acid-trace element (Zn, Cu, Mn) interactions. *International Journal of Food Science & Technology, 37*, 749–758.

Lopez, H. W., Coudray, Ch., Levrat-Verny, M. A., Feillet-Coudray, C., Demigné, C., & Rémésy, C. (2000). Fructooligosacharides enhance mineral apparent absorption and counteract the deleterious effects of phytic acid on mineral homeostasis in rats. *Journal of Nutrition Biochemistry, 11*, 500–508.

Lott, J. N. A., Greenwood, J. S., & Batten, G. D. (1995). Mechanisms and regulation of mineral nutrient storage during seed development. In J. Kigel & G. Galili (Eds.), *Seed development and germination* (pp. 215–235). New York: Marcel Dekker.

Lynch, S. R., Dassenko, S. A., Cook, J. D., Juillerat, M. A., & Hurrell, R. F. (1994). Inhibitory effect of a soybean–protein-related moiety on iron absorption in humans. *American Journal of Clinical Nutrition, 60*, 567–572.

Martinez, B., Rincón, F., & Ibáñez, M. V. (2000). Optimization of tannin extraction from infant foods. *Journal of Agriculture & Food Chemistry, 48*, 2097–2100.

Mernissi-Arifi, K., Wehrer, C., Schlewer, G., & Spiess, B. (1994). Complexation studies on inositol-phosphates. V. Cu^{2+}, Zn^{2+}, Fe^{2+} and Fe^{3+} complexes of some myo-inositol triphosphates. *Journal of Inorganic Biochemistry, 55*, 263–277.

Miller, D. D., Schricker, B. R., Rasmussen, R. R., & Van Campen, D. (1981). An in vitro method for estimation of bioavailability from meals. *American Journal of Clinical Nutrition, 34*, 2248–2256.

Minihane, A. M., & Rimbach, G. (2002). Iron absorption and the iron binding and anti-oxidant properties of phytic acid. *International Journal of Food Science & Technology, 37*, 741–748.

Morris, E. R., & Ellis, R. (1980). Effect of dietary phytate/zinc molar ratio on growth and bone zinc response of rats fed semipurified diets. *Journal of Nutrition, 110*, 1037–1042.

Myers, R. H., & Montgomery, D. C. (1995). Introduction to response surface methodology. In G. E. P. Box & D. W. Montgomery (Eds.), *Response surface methodology. Process and product optimization using designed experiments* (pp. 1–16). New York, NY: Wiley.

Persson, H., Nyman, M., Liljeberg, H., & Frølich, W. (1991). Binding of mineral elements by dietary fibre components in cereal-in vitro (III). *Food Chemistry, 40*, 169–183.

Persson, H., Türk, M., & Nyman, M. (1998). Binding of Cu^{2+}, Zn^{2+} and Cd^{2+} to inositol tri-, tetra-, penta- and hexaphosphates. *Journal of Agricultural & Food Chemistry, 46*, 3194–3200.

Plaami, S., & Kumpulainen, J. (1991). Determination of phytic acid in cereals using ICP-AES to determine phosphorus. *Journal of the Association of the Official Analytical Chemistry, 74*, 32–36.

Prosky, P., Aspm, N. G., Schweizer, T. F., Devries, J. W., & Furda, I. (1988). Determination of insoluble and soluble dietary fibre in food and food products: Collaborative study. *Journal of the Association of the Official Analytical Chemistry, 68*, 677–679.

Reddy, M. B., Hurrell, R. F., & Cook, J. D. (2000). Estimation of non-hemé bioavailability from meal composition. *American Journal of Clinical Nutrition, 71*, 937–943.

Rincón, F., Ros, G., Periago, M. J., & Martínez, C. (1996). Design of product as source of variance in composition of meat-based infant beikosts. *Meat Science, 43*, 99–109.

Ros, G., Abellán, P., Rincón, F., & Periago, M. J. (1994). Electrolyte composition of meat-based infant beikosts. *Journal of Food Composition & Analysis, 7*, 282–290.

Souci, S. W., Fachmann, W., & Kraut, H. (1994). Meat and organs of slaughtered animals. In S. W. Souci, W. Fachmann, & H. Kraut (Eds.), *Food composition and nutrition tables* (p. 193). Stuttgart, Germany: Medpharm GmbH Scientific Publishers.

Torre, M., Rodriguez, A., & Saura-Calixto, F. (1991). Effects of dietary fiber and phytic acid on mineral bioavailability. *Critical Review in Food Science & Nutrition, 1*, 1–22.

Vaquero, M. P., Van Dokkum, W., Bos, K. D., Wolters, M. G. E, Schaafsma, G., & Luten, J. B. (1992). In vitro availability of calcium, magnesium, iron, copper and zinc from white or brown breads separately or in combination with other foods. *Food Science & Technology International, 32*, 47–58.

Yoshida, K., Wada, T., Koyama, H., Mizobuchi, R., & Naito, S. (1999). Temporal and spatial patterns of accumulation of the transcript of myo-inositol-1-phosphate synthetase and phytin-containing particles during seed development in rice. *Plant Physiology, 119*, 65–72.